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Letter

Persistence of SARS-CoV-2 in Water and Wastewater

Aaron Bivins,[#] Justin Greaves,[#] Robert Fischer,[#] Kwe Claude Yinda, Warish Ahmed, Masaaki Kitajima, Vincent J. Munster, and Kyle Bibby*

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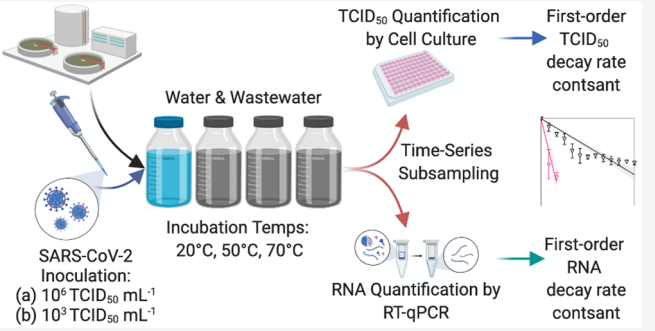
ABSTRACT: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA is frequently detected in the feces of infected individuals. While infectious SARS-CoV-2 has not previously been identified in wastewater, infectious SARS-CoV-2 has been isolated from the feces of at least one patient, raising concerns about the presence of infectious SARS-CoV-2 in wastewater. The fate and inactivation characteristics of SARS-CoV-2 in water and wastewater are unknown, with current inactivation estimates based on surrogate models. In this study, the persistence of SARS-CoV-2 infectivity and RNA signal was determined in water and wastewater. The times for 90% reduction (T_{90}) of viable SARS-CoV-2 in wastewater and tap water at room temperature were 1.5 and 1.7 days, respectively. In high-starting titer (10^5 $TCID_{50}$ mL^{-1}) experiments, infectious virus persisted for the entire 7-day sampling time course. In wastewater at 50 and 70 °C, the observed T_{90} values for infectious SARS-CoV-2 were decreased to 15 and 2 min, respectively. SARS-CoV-2 RNA was found to be significantly more persistent than infectious SARS-CoV-2, indicating that the environmental detection of RNA alone does not substantiate risk of infection.



INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a novel betacoronavirus, is the causative agent of the ongoing pandemic of coronavirus disease 2019 (COVID-19). Concomitant with respiratory infection, SARS-CoV-2 has been observed to infect the gastrointestinal tract via the angiotensin-converting enzyme (ACE) 2 receptor that is expressed by epithelial cells in the gastrointestinal system.[1] SARS-CoV-2 RNA has been detected for prolonged periods in the stool of a portion of individuals infected with SARS-CoV-2.[2] In addition to the reported detections of SARS-CoV-2 RNA in stool, intact SARS-CoV-2 virions have been observed in feces via electron microscopy[3] and a single study reported isolating infectious SARS-CoV-2 via cell culture.[4] These reports have raised concerns about the potential for SARS-CoV-2 to be transmitted by fecal−oral[5] or fecal−nasal pathways, as was strongly implicated for the SARS-CoV-1 Amoy Gardens outbreak in 2003[6] and recently suggested for SARS-CoV-2.[7] While SARS-CoV-2 RNA has been detected in untreated wastewater, primarily for disease surveillance applications known as wastewater-based epidemiology,[8−15] attempts to culture SARS-CoV-2 from wastewater originating from a hospital COVID-19 isolation unit[16] and domestic wastewater and river water positive for SARS-CoV-2 RNA[17] were not successful. The need to assess SARS-CoV-2 fate and stability in the water and wastewater in light of the COVID-19 pandemic has been widely discussed in the literature.[18−22]

The persistence of SARS-CoV-2 in water has been preliminarily described via observations of surrogates, such as bacteriophage Φ6 and other coronaviruses, including murine hepatitis virus (MHV), feline infectious peritonitis virus (FIPV), transmissible gastroenteritis virus (TGEV), and human coronaviruses (HCoV) 229E and OC43. A recent systematic review and meta-analysis of coronavirus persistence in water found that the persistence of these coronaviruses and the surrogates increased with a decrease in temperature with 99% reduction times increasing from approximately 2 days in raw wastewater at 22−25 °C (n = 14) to approximately 50 days in raw wastewater at 4−6 °C (n = 2).[23] Other reports of coronavirus persistence have varied; for example, in pasteurized settled sewage at 25 °C, TGEV and MHV required 9 and 7 days, respectively, for 99% reduction with <90% reduction observed for each after 4 weeks in the same matrix at 4 °C.[24] Alternatively, in a study using unpasteurized wastewater, infectious MHV was reduced by 90% in 13 h at 25 °C.[25] In primary effluent at 23 °C, FIPV and HCoV 229E demonstrated 99.9% reduction in 2 and 4 days, respectively.[26] Notably, the suitability of enveloped virus surrogates has been

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previously reported to be variable depending upon the environmental conditions,[27] demonstrating the need for confirmatory experiments with the pathogen of concern.

Despite the high public health interest and varying reports from surrogate experiments, no data about SARS-CoV-2 persistence in water and wastewater are available. The persistence of SARS-CoV-2 is important for characterizing the exposure risks associated with wastewater to the general public, wastewater utility personnel, and scientists working with wastewater as part of wastewater-based epidemiology. In the study presented here, we provide an analysis of the persistence of SARS-CoV-2 in representative municipal wastewater and tap water as determined by a cell culture infectivity assay and reverse transcription quantitative polymerase chain reaction (RT-qPCR).

MATERIALS AND METHODS

Previously described methods for assessing the persistence and disinfection of Ebola virus in sterilized wastewater and on surfaces were adopted for SARS-CoV-2.[28−30] On August 5, 2019, approximately 1 L of untreated primary influent was collected from a municipal wastewater treatment plant in northern Indiana, United States, receiving an average flow of 11 million gallons per day (MGD). Immediately after being collected, the sample was stored overnight at −80 °C and on August 6, 2019, shipped to the Rocky Mountain Laboratories (RML) overnight on ice. The sample was then stored at −80 °C at RML until the experiments described herein were begun. The wastewater was not sterilized prior to the experiments with SARS-CoV-2.

All SARS-CoV-2 cultivation experiments were performed at RML in BSL4 laboratories. Stock virus, SARS-CoV-2 nCoV-WA1-2020 (MN985325.1), isolated from a clinical patient diagnosed with COVID-19,[31] was cultured on high-passage Vero E6 cells. The harvested virus was centrifuged to separate the virus from cellular debris, and the final resulting titer determined by titration was 10^6 median tissue culture infectious dose per milliliter ($TCID_{50}$ mL^{-1}). To assess its long-term stability, the virus was inoculated into two separate 15 mL wastewater volumes to achieve starting titers of approximately 10^5 and 10^3 $TCID_{50}$ mL^{-1}. We note that the viral concentrations employed were increased over expected possible infectious SARS-CoV-2 concentrations in sewage to enable the determination of inactivation kinetics. Aliquots of 5 mL from each wastewater volume were then distributed into three separate 15 mL vials to perform decay experiments in triplicate for wastewater at 20 °C [room temperature (RT)], 50 °C, and 70 °C and tap water (inoculated at 10^5 $TCID_{50}$ mL^{-1}) at room temperature. Samples were collected at 1, 4, and 8 h time points in the first 24 h and daily up to 7 days. At each time point, including immediately following inoculation, 100 μL of a briefly agitated sample was removed from the bulk sample vial and added to 900 μL of Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with heat-inactivated fetal bovine serum (FBS, Gibco) and 2% penicillin/streptomycin (Gibco) to final concentrations of 50 units mL^{-1} penicillin and 50 μg mL^{-1} streptomycin, and L-glutamine (Gibco) to a final concentration of 2 mM, and frozen at −80 °C. Negative controls consisting of 100 μL of uninoculated wastewater added to 900 μL of DMEM were also prepared at each time point.

To assess the potential effectiveness of heat-treating wastewater for biosafety, such as pasteurizing samples, and the potential of heat treatment for sludge, spiked wastewater was subjected to heat treatment at 50 and 70 °C. Stock virus SARS-CoV-2 nCoV-WA1-2020 (MN985325.1) was diluted 1:10 in wastewater, and 1 mL aliquots were pipetted into 2 mL screw-top vials with three replicate vials for each time point in the experiment. To ensure more efficient heat transfer, 1 mL of water was added directly into each well in the Eppendorf heat block. Once the heat block and the water within each well reached the target temperature, the 36 closed vials containing the inoculated wastewater were inserted into the heat block wells. Three vials were removed from the heat block and immediately placed in a −80 °C freezer at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 min. A time zero sample was prepared in the same manner but never placed into the heat block and instead immediately frozen at −80 °C. Because of the limited heat block capacity, the 50 and 70 °C experiments were performed asynchronously; however, fresh wastewater vials, including time zero samples, were prepared immediately prior to each temperature experiment.

To determine the virus titer, each 1 mL sample was thawed and four 10-fold dilution series per sample were created by passing 20 μL of the sample into 180 μL of DMEM. A 96-well culture plate seeded with Vero E6 cells was inoculated with a 100 μL volume from the dilution series such that an end-point dilution with an expected limit of detection of approximately 5.6 $TCID_{50}$ per milliliter would result. The culture plate was incubated with the virus dilutions for 1 h, then the medium removed from the two highest titers rinsed twice with phosphate-buffered saline (PBS), and 100 μL of fresh culture medium added to the two highest titers. The culture plate was then incubated at 37 °C for 5 days, inspected for cytopathic effect (CPE), and scored, and $TCID_{50}$ was calculated according to the Spearman−Karber method[32] for each matrix and temperature at each sampling time point in triplicate. No CPE was observed in the negative controls (DMEM with uninoculated wastewater) included in the study.

To determine the stability of the SARS-CoV-2 RNA signal in wastewater, the samples were assayed by RT-qPCR. The samples were first inactivated using the standard operating procedure (SOP) for removing samples from the BSL4 laboratory. Briefly 140 μL of the sample was added to 560 μL of buffer AVL (Qiagen) and incubated at RT for 10 min. The entire contents were then added to 560 μL of absolute ethanol and incubated at RT for an additional 10 min prior to being moved to the BSL2 laboratory. The RNA was extracted using a QIAamp 96 Virus QIAcube HT Kit with a QIAvac 96 vacuum system (Qiagen) following the manufacturer's instructions. The RT-qPCR assay used targets the E gene of SARS-CoV-2.[33] Briefly, extracted RNA was measured using QuantiFast Probe RT-PCR + ROX Vial Kit (Qiagen) reagents in a Rotor-Gene Q (Qiagen) real-time thermocycler. Cycling conditions were as follows: reverse transcription for 10 min at 50 °C, denaturation and activation for 5 min at 95 °C, and two-step cycling 10 s at 95 °C and 30 s at 60 °C for 40 cycles. The primer/probe set used for the E gene assay is the same as that used in the Berlin assay.[33] Standard curves were constructed using *in vitro*-transcribed RNA of known quantities for RNA quantification.

The decay of infectious SARS-CoV-2 in wastewater and water was analyzed using both monophasic (first-order) and biphasic decay models. Data that fit a monophasic decay model can be transformed to describe a line with a slope, k, the first-order decay rate constant, per eq 1:

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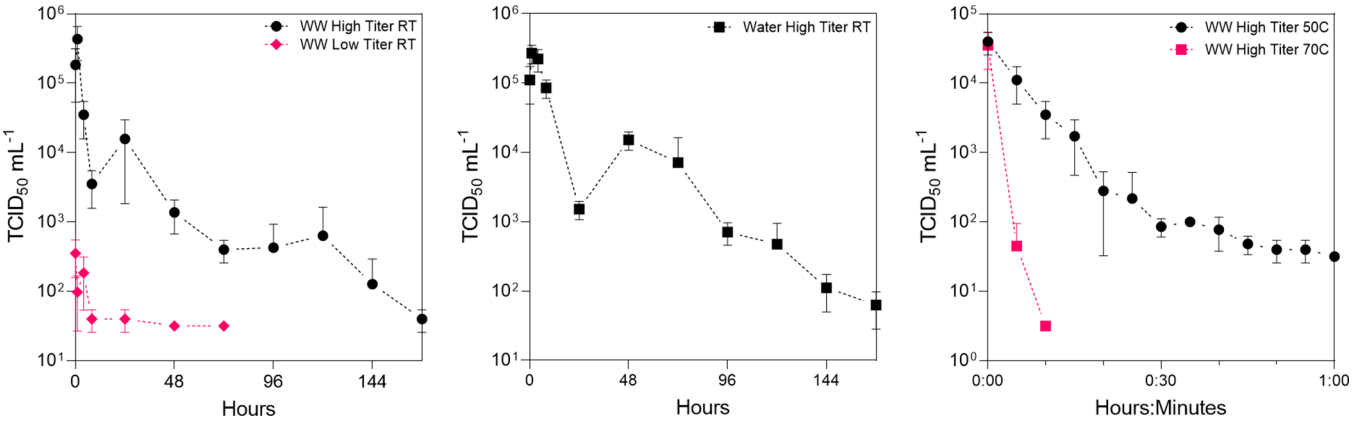

Figure 1. Observed $TCID_{50}$ per milliliter decay (mean and standard deviation) in wastewater (WW) inoculated with high (10^5 $TCID_{50}$ per milliliter) and low (10^3 $TCID_{50}$ per milliliter) titers of infectious SARS-CoV-2 at room temperature (20 °C, RT) (left), water inoculated with a high titer at RT (middle), and WW inoculated with a high titer at 50 and 70 °C (right). Mean and standard deviation are displayed. Where the standard deviation resulted in negative values, single-sided error bars are displayed. Where error bars are not shown the standard deviation is too small to display.

Table 1. First-Order Decay Rate Constants (k), Half-Lives, and Decimal Reductions for Infectious SARS-CoV-2 in Wastewater (WW) Inoculated with High and Low Titers at Room Temperature (20 °C, RT), with a High Titer in Tap Water at RT, and with a High Titer in WW at 50 and 70 °C as Estimated by Linear Regression of Transformed $TCID_{50}$ Measurements[a]

	WW, high titer, RT (0−7 days)	WW, low titer, RT (0−3 days)	tap water, high titer, RT (0−7 days)	WW, high titer, 50 °C (0−60 min)	WW, high titer, 70 °C (0−10 min)
n	33	21	33	39	9
k_{mean} (95% CI)	1.4 $days^{-1}$ (1.3−1.6 days)	1.1 $days^{-1}$ (0.71−1.5 days)	1.2 $days^{-1}$ (1.1−1.3 days)	0.15 min^{-1} (0.14−0.16 min)	1.0 min^{-1} (0.80−1.3 min)
r^2	0.71	0.54	0.88	0.68	0.88
RMSE	1.8	1.2	1.2	1.4	1.9
half-life mean (95% CI)	0.49 day (0.43−0.56 day)	0.64 day (0.48−0.98 day)	0.59 day (0.54−0.66 day)	4.6 min (4.3−5.1 min)	0.67 min (0.55−0.86 min)
T_{90} mean (95% CI)	1.6 days (1.4−1.8 days)	2.1 days (1.6−3.3 days)	2.0 days (1.8−2.2 days)	15 min (14−17 min)	2.2 min (1.8−2.9 min)
T_{99} mean (95% CI)	3.2 days (2.9−3.7 days)	4.3 days (3.2−6.5 days)	3.9 days (3.6−4.4 days)	30 min (28−34 min)	4.5 min (3.7−5.7 min)

[a]The time frames of the measurement used in the estimations are shown in parentheses within each column header.

$$\ln\left(\frac{C_t}{C_0}\right) = -kt \quad (1)$$

where C_t is the concentration of the virus at time t and C_0 is the starting virus concentration at time zero. The mean first-order decay rate constant and its 95% confidence interval (95% CI) in units of inverse time are estimated by linear regression. The biphasic decay model is described per eq 2 with two different periods of exponential decay, a "fast" period and a "slow" period:

$$C_t = C_{f0}e^{-k_1 t} + C_{s0}e^{-k_2 t} \quad (2)$$

where C_t is the concentration of microbes at time t, k_1 is the initial and fast decay rate, k_2 is the final and slow inactivation rate, and C_{f0} and C_{s0} are the initial concentrations of infectious virus at the start of the fast and slow decay periods, respectively. Both monophasic and biphasic models were fit to the observed data and compared using an extra sum-of-squares F test. The decay of SARS-CoV-2 RNA in wastewater and water was analyzed using only a monophasic decay model. To assess the change in the decay rate constant with temperature in wastewater, the estimated mean values of k at 20, 50, and 70 °C were $\log_{10}$ transformed and a linear regression was performed. All plotting, regressions, and statistical analyses were performed in GraphPad PRISM ver. 8.0 (GraphPad, San Diego, CA).

RESULTS AND DISCUSSION

Monophasic versus Biphasic Decay Models. The physicochemical characteristics of the primary influent wastewater used in the decay experiments are summarized in Table S1. The observed decay in infectious SARS-CoV-2 for each experimental condition is displayed in Figure 1. A comparison of the monophasic and biphasic decay models using the extra sum-of-squares F test found that biphasic decay did not improve the fit of the model to the observed data over monophasic decay ($p > 0.05$) for infectious SARS-CoV-2 in wastewater and water (Table S2). For this reason, only the first-order decay models, the rate constants, and the associated decimal reduction times were further analyzed.

Infectivity First-Order Decay Rate Constants and Decimal Reduction Times. As shown in Figure 1, infectious SARS-CoV-2 could be detected for the entire 7 day duration of the high-titer (10^5 $TCID_{50}$ mL^{-1}) experiments and for 3 days of the low-titer (10^3 $TCID_{50}$ mL^{-1}) experiments. Figure S1 displays the observed infectivity time series, linearized as described in eq 1 with the slopes and 95% confidence regions as fit by linear regression. As summarized in Table 1, at room temperature, the estimated mean first-order decay rate constants were 1.4 $days^{-1}$ in wastewater at high titer, 1.1 $days^{-1}$ in wastewater at low titer, and 1.2 $days^{-1}$ in tap water at high titer. We note that the high- and low-titer decay constants in wastewater were not statistically different (Mann−Whitney

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p value >0.05), while at 50 and 70 °C, mean rate constants in wastewater were 0.15 and 1.0 min^{-1}, respectively. On the basis of these rate constants, the times for a 90% reduction in titer (T_{90}) were estimated to be between 1.6 and 2.1 days in wastewater at room temperature (high- and low-titer experiments, respectively), 2.0 days in tap water at room temperature, and 15 and 2.2 min in wastewater at 50 and 70 °C, respectively. Mean and 95% CI for the estimated first-order decay rate constants, half-life, T_{90}, and T_{99} for each experiment are summarized in Table 1.

The persistence observed for infectious SARS-CoV-2 in untreated wastewater varied from estimates of the persistence of other coronaviruses. The estimated T_{90} for infectious SARS-CoV-2 in wastewater at room temperature (20 °C) of 1.6−2.1 days is shorter than the 4 and 3 days previously observed for TGEV and MHV, respectively, in pasteurized and settled wastewater at 25 °C.[24] Alternatively, the estimated T_{90} is longer than the time of 13 h at 25 °C for MHV and 28 h at 10 °C for bacteriophage phi6, a coronavirus surrogate, in unpasteurized wastewater.[25] A linear regression of the $\log_{10}$-transformed rate constants in untreated wastewater versus incubation temperature, as shown in Figure 2, results in a mean slope of 0.07 and a y-intercept of −2.5 (r^2 = 0.98). A comparison to a similar regression performed for coronaviruses in a systematic review by Silverman and Boehm, also shown on Figure 2, indicates that the first-order decay rate constant for infectious SARS-CoV-2 shows a similar sensitivity to temperature (comparable slope), but that SARS-CoV-2 is more persistent than other coronaviruses in unsterilized wastewater at a given temperature (lower y-intercept).[23]

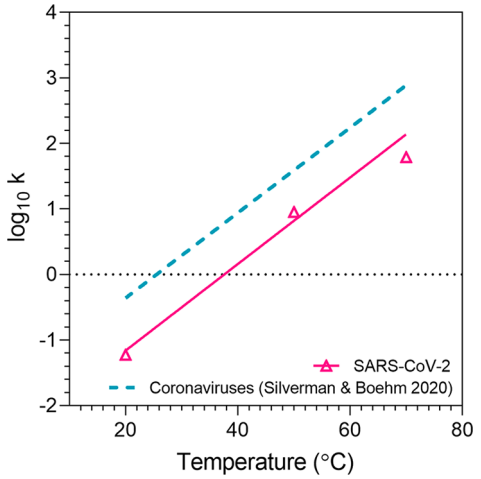

Figure 2. Linear regression of the $\log_{10}$-transformed mean first-order decay rate constants (in units of inverse hours) estimated for the decay of infectious SARS-CoV-2 in unsterilized wastewater at 20, 50, and 70 °C. The resulting line (r^2 = 0.98) was characterized by a slope of 0.07 (95% CI from 0.06 to 0.07) and a y-intercept of −2.5 (95% CI from −2.4 to −2.6). The linear regression from a systematic review and meta-analysis of coronavirus decay by Silverman and Boehm[23] is shown for reference.

RNA First-Order Decay Rate Constant and Decimal Reduction Times. In addition to infectious SARS-CoV-2, the time series of observed SARS-CoV-2 RNA was also determined for wastewater and water incubated at room temperature. Figure S2 displays the transformed RNA data, linear regressions, and associated 95% confidence regions. First-order decay rate constants were estimated for RNA (Table S3). Estimated decay rate constants indicate that the SARS-CoV-2 RNA signal is more persistent than infectious SARS-CoV-2 with T_{90} values of 3.3 days versus 1.6 days in wastewater at high titer, 26.2 days versus 2.1 days at low titer, and 33.2 days versus 2.0 days for tap water. The observed T_{90} of low-copy number SARS-CoV-2 RNA in untreated wastewater at 20 °C in this study is greater than, but comparable to, the T_{90} values for untreated wastewater at 15 and 25 °C, 20.4 and 12.6 days, respectively, reported during a study using gamma-irradiated SARS-CoV-2.[34] The observed low-copy number persistence of SARS-CoV-2 RNA in wastewater is also comparable with the reported T_{90} of 21 days for Zika virus in untreated sewage at 25 °C.[35] While the T_{90} estimated for the high-copy number experiment is much shorter than the RNA persistence from each of these studies, the RNA copy number was remarkably stable from 72 to 168 h during the experiment. Importantly, we continued to detect SARS-CoV-2 RNA, even when infectious SARS-CoV-2 was below the detection limit of the cell culture assay, which implies that the detection of RNA alone may not be a specific indication of infectious virus. We note that r^2 values of the regression fit to the SARS-CoV-2 RNA decay were poor due to variation among the replicates; however, these data clearly demonstrate that the RNA signal is more persistent under the tested conditions than infectious virus.[36] Recently, a publication reported circumstantial evidence of fecal aerosol transmission of SARS-CoV-2 in a high-rise apartment building, including the detection of RNA in environmental samples.[7] Another study has reported being unable to culture infectious SARS-CoV-2 from wastewater despite detecting RNA from the virus.[17] However, it is possible that the failure to culture SARS-CoV-2 from wastewater could be due to the concentration methods used, which may inactivate virus particles or co-concentrate substances toxic to cell culture.[37] Despite these conflicting reports, our observations during the persistence experiments suggest that detection of SARS-CoV-2 RNA alone in wastewater does not suffice to characterize the risk of infection attributable to exposure to that wastewater.

Limitations and Interpretation. The scope of the study was limited by the inherent constraints of working with infectious SARS-CoV-2 and access to the necessary experimental resources. The experiments conducted made use of frozen and thawed wastewater, which may have altered the microbiota that contribute to the decay of infectious virus particles in the wastewater matrix.[38,39] Because the experiments made use of influent from only a single treatment plant, they could not examine other environmental factors that can vary in wastewater and contribute to variability in the decay of infectious virus, such as pH, suspended solids, and mixing conditions.[25,27] Additionally, the wastewater and water were inoculated with virus titers that are likely higher than would be expected in real world scenarios to enable the observation of the viral decay rate using the methods available. Importantly, during the low-titer inoculation wastewater experiments at 20 °C, the observed $TCID_{50}$ per milliliter was below the cell culture limit of detection after only 72 h. The linear regressions used to estimate the first-order decay rate constant for this experiment do not include the censored data beyond 72 h. While biphasic decay did not result in an improved model fit, apparent tailing of the decay curve may suggest behavior outside of the assumed first-order decay, for example, due to particle association of the virus. Future cell culture experiments with lower limits of detection should be used to explore this in

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more detail. Applications of the estimated decay parameters, especially at low titers, should account for the uncertainty and variability inherent in biological systems as appropriate for the context.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.estlett.0c00730.

Physicochemical characteristics of untreated wastewater used in this study (Table S1), extra sum-of-squares F test results for biphasic versus monophasic decay models of infectious SARS-CoV-2 (Table S2), linearized $TCID_{50}$ measurements (Figure S1), linearized SARS-CoV-2 RNA gene copy measurements (Figure S2), and first-order decay rate constants for SARS-CoV-2 RNA (Table S3) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Kyle Bibby − *Department of Civil & Environmental Engineering & Earth Sciences, University of Notre Dame, Notre Dame, Indiana 46556, United States;* orcid.org/0000-0003-3142-6090; Email: kbibby@nd.edu

Authors

Aaron Bivins − *Department of Civil & Environmental Engineering & Earth Sciences and Environmental Change Initiative, University of Notre Dame, Notre Dame, Indiana 46556, United States*

Justin Greaves − *Department of Civil & Environmental Engineering & Earth Sciences, University of Notre Dame, Notre Dame, Indiana 46556, United States*

Robert Fischer − *Laboratory of Virology, Rocky Mountain Laboratories (RML), National Institutes of Health, Hamilton, Montana 59840, United States*

Kwe Claude Yinda − *Laboratory of Virology, Rocky Mountain Laboratories (RML), National Institutes of Health, Hamilton, Montana 59840, United States*

Warish Ahmed − *CSIRO Land and Water, Dutton Park, QLD 4102, Australia*

Masaaki Kitajima − *Division of Environmental Engineering, Hokkaido University, Sapporo, Hokkaido 060-8628, Japan;* orcid.org/0000-0002-8142-511X

Vincent J. Munster − *Laboratory of Virology, Rocky Mountain Laboratories (RML), National Institutes of Health, Hamilton, Montana 59840, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.estlett.0c00730

Author Contributions

#A.B., J.G., and R.F. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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